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Unique Sensing Interface That Allows the Development of an Electrochemical Immunosensor for the Detection of Tumor Necrosis Factor α in Whole Blood

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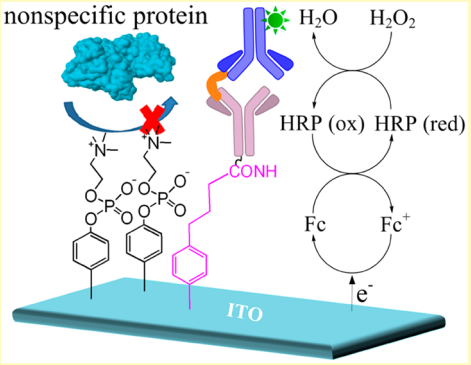
Supporting Information

ABSTRACT: Electrochemical affinity biosensors that can operate in whole blood are a rarity because biofouling of electrode surfaces compromises the performance of the final device. The common anti-biofouling layers that can be applied to electrodes, poly(ethylene glycol) (PEG) or oligo(ethylene glycol) (OEG) layers, form a high impedance layer on the electrode, effectively passivating the electrode. In response to this issue, we have developed effective anti-biofouling chemistry, that employs short chain zwitterionic species, derived from aryl diazonium salts, that give low impedance layers compatible with amperometry. Herein, we demonstrate the application of this surface chemistry to mixed layers of phenyl phosphorylcholine (PPC) and phenyl butyric acid (PBA), to develop immunosensors that can be used in whole blood. The capability of these new modification layers is demonstrated with an immunosensor for detecting tumor necrosis factor α in whole blood. The immunosensor is shown to specifically and precisely detect TNF-α in whole blood samples with a minimum detection limit of 10 pg/mL with a wide linear range of 0.01 ng/mL to 500 ng/mL. The results are comparable with those from commercial ELISA kit, indicating the developed immunosensor has great potential for future clinic use.



KEYWORDS: *mixed layers, aryldiazonium salts, electrografting, affinity biosensor, antifouling*

Developing electrochemical biosensors with interfacial design that achieves high sensitivity and selectivity continues to be a challenge for detecting proteins in complex biological samples despite the promise for the early detection of diseases.[1−3] The challenge arises from the abundance of proteins that exist in bodily fluids which nonspecifically adsorb on the sensing interface. The result of this biofouling is changes in sensitivity, specificity, irreproducibility of response, and even complete failure of the sensing devices.[4−8] Effective solutions are needed that resist this nonspecific protein adsorption. The inhibition of nonspecific protein adsorption is most commonly achieved by modifying the surface with highly hydrophilic poly(ethylene glycol) (PEG) polymer brushes,[9−11] oligo (ethylene glycol) (OEG) contained self-assembled monolayers (SAMs),[12,13] and OEG layers derived from aryl diazonium salts.[14−16] These layers have excellent antifouling properties due to formation of a hydrated layer by hydrogen bonding between the ethylene glycol units and water molecules.[17]

As good as these molecules are, their long-chain organic layers (OEG and PEG) are not desirable for many electrochemical methods because they form a high-impedance layer, which prevents faradaic electrochemistry from occurring with the underlying electrode.[18] The most common alternative strategy employed is "backfilling" of nonsensing regions in the biointerface with bovine serum albumin (BSA) to provide resistance of nonspecific protein adsorption to the sensing interfaces.[19,20] This strategy also gives high impedance layers, and there is concern about the dynamic nature of these protein layers, which can influence the reproducibility and reliability of a sensor.[7,21,22]

A solution to forming anti-biofouling layers that are not high impedance comes from recent active exploration of short chain zwitterionic species containing phosphorylcholine (PC) moiety which confers to surfaces high resistance to protein adsorption.[18,23−26] Related to this solution is the development of the application of reductive electrografting of aryldiazonium salts derived layers onto conducting or semiconducting surfaces which can give robust, thick films on electrodes that do not completely passivate the electrode from performing faradaic electrochemistry.[27−30] Our group has investigated the combination of these ideas by modifying of glassy carbon, gold, and indium tin oxide (ITO) electrodes by electrografting the

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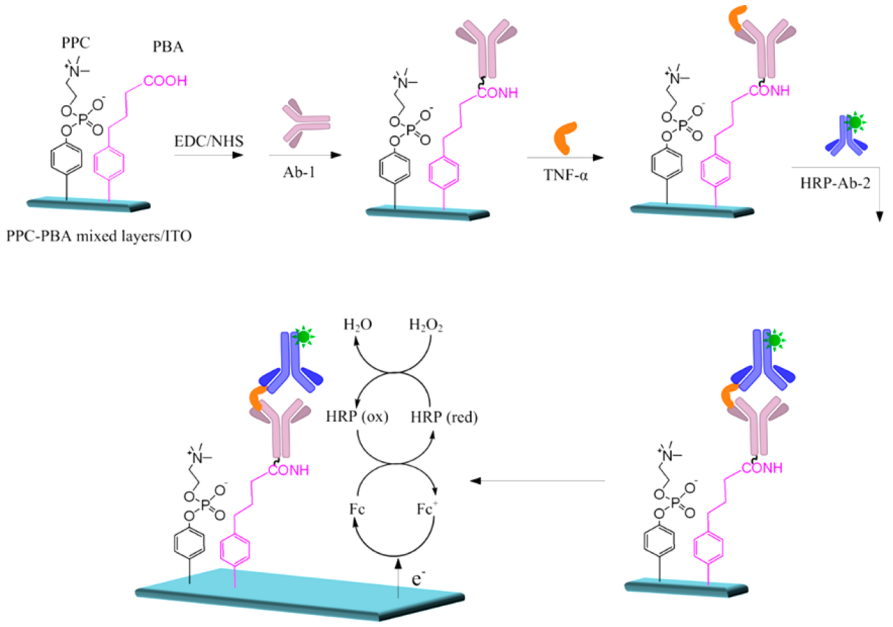
Scheme 1. Illustration of the PPC−PBA Mixed Layers/ITO Based Immunosensor for the Detection of TNF-α[a]



[a]The sensing principle is based on an enzyme-dependent catalytic current response of H_2O_2 which originates from the HRP reaction, and the soluble redox mediator of ferrocenemethanol could effectively shuttle electrons from the base electrode.

aryldiazonium salts of phenyl phosphorylcholine (PPC).[18,24,25] The resultant PPC layer exhibits exceptionally low anti-biofouling performance with sufficiently low impedance that significant electron transfer can proceed across the layer.[24] To make this surface chemistry applicable to biosensing, methods for the electrodeposition of mixed layers containing PPC and a minor amount of carboxyphenyl species for attaching biomolecules have been developed.[23] Such mixed-layer surfaces have the advantages of (1) low impedance, (2) anti-biofouling capability, and (3) controllable surface composition where the biorecognition species are located in an anti-biofouling layer for the selective detection of the molecule of interest.[5,23,31−33]

In this paper, a mixed layer surface chemistry is developed further to allow affinity biosensing applications in complex samples. Herein, we demonstrate mixed layers of zwitterionic phenyl phophorylcholine (PPC) and phenyl butyric acid (PBA), in which PPC takes responsibility to repel nonspecific protein adsorption while PBA allows the bioconjugation of antibodies to the electrode as the biorecognition elements. To demonstrate the sensing capability of this electrode interface, the detection of tumor necrosis factor α (TNF-α) in undiluted whole blood was performed using a "sandwich" assay format (Scheme 1). TNF-α is a 17.5 kDa pro-inflammatory cytokine that can mediate a variety of biological effects such as immune regulation, antitumor activity, viral replication, and infection resistance.[34−36] It is at a low level (pg/mL range) in healthy human blood, whereas a several-fold increase is observed in septic patients.[37] Overproduction of TNF-α occurs in numerous pathological conditions, including cancer, heart disease, diabetes, and autoimmune diseases. This allows TNF-α to be a typical early stage indicator of an inflammatory reaction, in response to infection or cancer.[38,39] Therefore, the development of sensitive and specific assay methods for the detection of the trace biomarkers is very important for the understanding of tumor biological process, inherent mechanism, and drug discovery, and it has a therapeutic potential for the treatment of diseases.[40,41] Analytical criteria with low detection limit (~11 pg/mL), dynamic range (5−44.4 pg/mL), and fast response time (seconds to minutes) must be taken into consideration for the immunosensor development.[37,44]

EXPERIMENTAL SECTION

Reagents and Materials. Dichloromethane, methanol, K_2CO_3, $NaNO_2$, KCl, NaCl, HCl (32%), H_2O_2 (30%), KH_2PO_4, and Na_2HPO_4 were purchased from Ajax Finechem (Sydney, Australia). 4-Aminophenyl phosphorylcholine (PPC) was obtained from Toronto Research Chemicals (Toronto, Canada). Ferrocenemethanol (97%), 4-(4-aminophenyl) butyric acid (PBA), hemoglobin (Hb), human serum albumin (HSA), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were acquired from Sigma (Sydney, Australia). TNF-α full-length protein (ab9642), anti-TNF α antibody (Ab1), and horseradish peroxidase conjugated anti-TNF α antibody (HRP-Ab2, ab24473) were ordered from Abcam (Melbourne, Australia). ITO coated glass slides (8−12 ohm/sq) were obtained from Delta Technologies (Loveland, USA). Phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4 and 10 mM Na_2HPO_4 with pH 7.4, and other aqueous solutions were prepared using Milli-Q water (18 MΩ cm^{-1}, Millipore, Sydney, Australia). Whole blood samples (anticoagulated with K2 EDTA) were acquired from Innovative Research (Novi, USA).

Fabrication of PPC−PBA Mixed Layers on ITO. Prior to surface modification, ITO electrodes were cleaned by successive ultrasonication in dichloromethane (DCM, 10 min), methanol (10 min), 0.5 M K_2CO_3 in 2:1 (volume ratio) methanol:H_2O (30 min), and copious amounts of Milli-Q water. The surfaces then were dried using nitrogen. PPC−PBA aryldiazonium salt mixture solution was generated by preparing a 0.1 M HCl solution containing 900 μM PPC and 100 μM PBA with 1 mM $NaNO_2$ and kept in an ice bath with argon purging for 30 min. The electrodes were then modified with PPC−PBA mixed layers by holding depositing potential at −0.65 V vs Ag/AgCl for 2 min in a solution of the PPC−PBA aryldiazonium salt mixture.

Electrochemical Apparatus. All electrochemical measurements were performed using an Autolab potentiostat (Metrohm Autolab B.V., Netherlands) except when electrochemical impedance spectroscopy (EIS) measurements were carried out, where a Solartron SI 1287

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with an SI 1260 frequency response analyzer (Solartron Analytical, Hampshire, England) was used. The electrochemistry was performed in a custom manufactured, 2.0 mL electrochemical cell in the three-electrode format with an Ag/AgCl (3.0 KCl) reference electrode and a platinum wire auxiliary electrode. The O-ring of the cell defined an electrode surface area of 0.24 cm^2.

X-ray Photoelectron Spectroscopy Characterization. XPS performed with an EscaLab220-IXL spectrometer using a monochromated Al Ka source (1486.6 eV), hemispherical analyzer, and multichannel detector. The spectra were taken with a 0.79 mm^2 spot size and a takeoff angle of 90° using a pressure of less than 1×10^{-8} mbar. The graphite C 1s (284.6 eV) was used as a reference for binding energies. Analysis of the XP spectra was conducted using the Avantage software. Background subtraction was performed with the Shirley routine followed by a nonlinear least-squares fitting to a mixed Gaussian−Lorentzian function. The sensitivity factors of each element, combined with their peak area, was used to determine the atomic percentage (atom %) of each element according to eq 1:

$$\text{atom\%} = \frac{A_i/S_i}{\sum A_i/S_i} \quad (1)$$

where A_i is the area of the element i, and s_i is the sensitivity factor for this element. The sensitivity factors for P 2p, O 1s, C 1s, and N 1s are 1.19, 2.93, 1.00, and 1.80, respectively.

Immunoassay Development. The capture antibody (Ab1) was immobilized onto the PPC−PBA/ITO surface via the classical EDC/NHS conjugation reactions between −COOH groups on the mixed-layer surface and residual amino groups of the Ab1. Specifically, the PPC−PBA/ITO surfaces were activated by immersion in 0.01 M PBS (pH 7.4) containing 25 mg/mL EDC and 30 mg/mL NHS for 1 h at room temperature. Subsequently, 40 μL of 10 μg/mL Ab1 was spread onto the resulting electrode surface to allow incubation at 4 °C in an ice bath for 1 h. Next, 40 μL of TNF-α with a series of concentrations (at each concentration of TNF-α, measurements were done at three individually fabricated electrodes) was dropped onto the Ab1 modified surfaces and incubated at room temperature for 1 h. After binding between Ab1 and TNF-α, the electrodes were finally incubated in 40 μL of 5 μg/mL HRP conjugated detection antibody (HRP-Ab2) to obtain the final sensing interface of HRP-Ab2/TNF-α/Ab1/PPC−PBA/ITO. The electrodes were rinsed thoroughly with PBS after each step. To test the specificity of developed immunosensor, hemoglobin (Hb, 5 μg/mL) and human serum albumin (HSA, 5 μg/mL) were used as interfering proteins. In whole blood assay, 40 μL of blood instead of standard TNF-α was added into Ab1/PPC−PBA/ITO interface; other steps were the same as the protocol discussed earlier.

Electrochemical measurements were carried out in 1.0 mL of 0.01 M PBS (pH 7.4) containing 2.0 mM H_2O_2 and 0.1 mM ferrocenemethanol as redox mediators with an applied potential of −0.05 V vs Ag/AgCl.

Determination of Protein Resistance. EIS and cyclic voltammetry (CV) were used to ascertain the ability of the final immunosensing interface to prevent nonspecific protein adsorption using a redox probe solution of 0.1 M KCl containing 1 mM $K_3[Fe(CN)_6]$ and 1 mM $K_4[Fe(CN)_6]$. The final sensing interfaces (HRP-Ab2/TNF-α/Ab1/PPC−PBA/ITO) were washed with copious amounts of water prior to incubating the surface in the solution for electrochemical analysis. The electrodes were then rinsed with water, before transfer to a solution containing the redox probe solution and 1 mg/mL HAS for EIS measurement. After incubation for 1 h, EIS was performed again. EIS spectra were recorded in the frequency range of 10^5 to 10^{-1} Hz. An AC potential with 0.01 V peak to peak separation was superimposed on a DC potential of 0.212 V. Impedance data were recorded and analyzed using ZPlot and ZView 3.1 software (Scribner Associates, Inc.), respectively. CV was carried out using the same redox probe solution and the same procedure of EIS with a scan rate of 100 mV/s.

RESULTS AND DISCUSSION

Formation of PPC−PBA Mixed Layers on ITO. First, the reductive adsorption of each aryldiazonium salt alone was explored, and thereafter a mixture of the two. It is important to note that the choice of PBA, distinct from the carboxyphenyl derivative we used previously,[23] was because the similarity in reductive adsorption potential of PPC and PBA made forming mixed layers of controllable surface composition potentially simpler. The cyclic voltammetry performed in a 1 mM PPC diazonium salt solution on the ITO surface showed one irreversible reduction peak at −0.55 V (Figure 1a) in the first

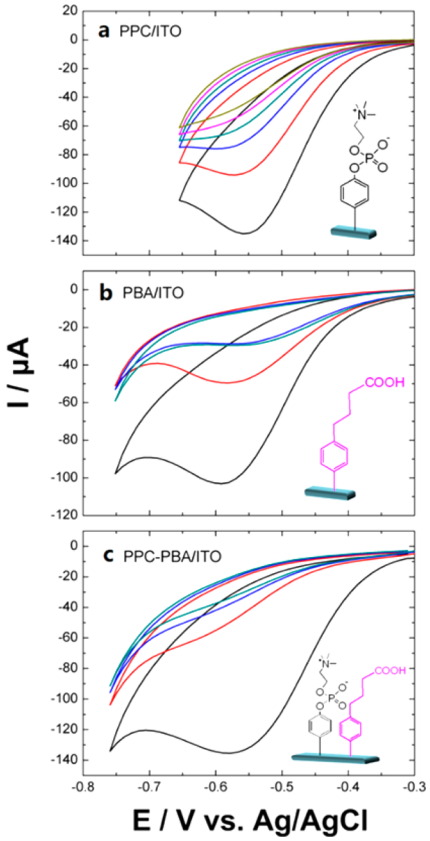

Figure 1. Cyclic voltammograms for reductive adsorption of aryldiazonium salts on ITO electrode from (a) 1 mM 4-aminophenyl phosphorylcholine (PPC) in 0.1 M HCl, (b) 1 mM 4-(4-aminophenyl)butyric acid (PBA) in 0.1 M HCl, and (c) 1 mM mixture of PBA and PPC (1:1 molar ratio) in 0.1 M HCl, with 1 mM $NaNO_2$ at a scan rate of 0.1 V/s.

cathodic scan. The PBA diazonium salt shows a similar reduction potential at −0.58 V (Figure 1b). As expected, the electrochemistry of the mixture of PPC−PBA (Figure 1c) reveals a single reduction peak at −0.56 V in the first cycle, which is somewhat broader than either of the single diazonium salt solutions.

Mixed layers of PPC−PBA on ITO were prepared using a fixed potential of −0.65 V for 2 min and XPS was used to examine the surface chemical composition (Figure 2). The characteristic peaks of phosphorus (~133.8 eV), quaternary nitrogen (~403 eV) from phosphorylcholine moiety of PPC, and carbon of the carboxylic acid group (~289.0 eV) from PBA were observed, indicating successful electrografting of mixed layers of PPC and PBA onto ITO electrode. The azo group located at around 399.7 eV is quite common in the case of

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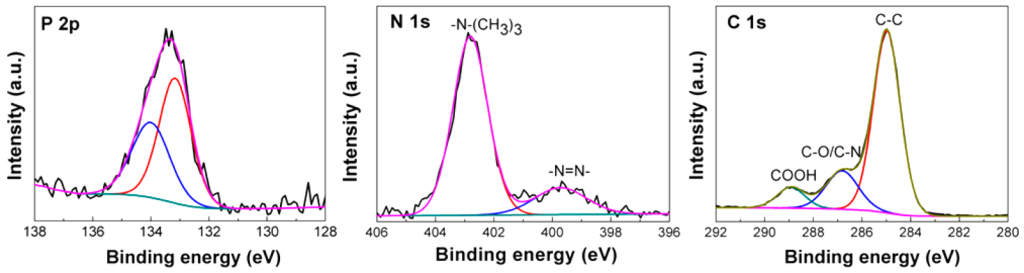

Figure 2. P 2p, N 1s, and C 1s core level XPS spectra for PPC−PBA mixed layer modified ITO. Reductive adsorption was performed in 0.1 M HCl solution containing PPC−PBA at molar ratio of 9:1 (total concentration is 1 mM). The potential and time during the reductive adsorption were −0.65 V vs Ag/AgCl and 2 min, respectively.

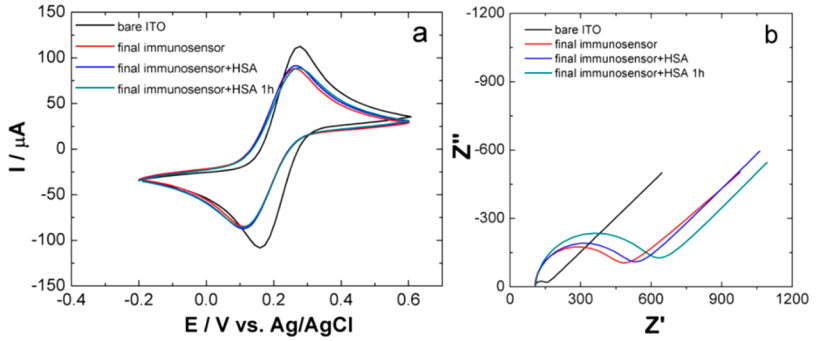

Figure 3. Examination of antifouling property using (a) cyclic voltammetry and (b) electrochemical impedance spectroscopy. Black line and red line represent bare ITO and final immunosensor with redox probe solution of $[Fe(CN)_6]^{3-/4-}$, respectively. The blue line and dark cyan line represent the final immunosensor after addition of redox probe solution containing1 mg/mL HSA, and incubation in this redox probe solution containing 1 mg/mL HSA for 1 h.

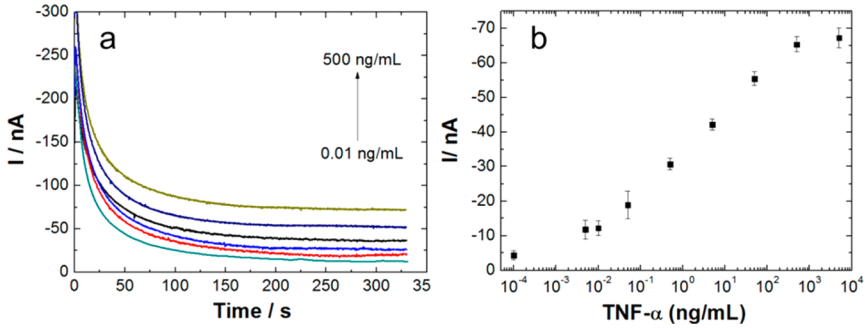

Figure 4. (a) Chronoamperometric current response of the immunosensor toward concentrations of TNF-α ranging from 0.01 ng/mL to 500 ng/mL (0.01, 0.05, 0.5, 5, 50, 500, ng/mL). (b) Calibration plot of the PPC−PBA mixed layers based immunosensor toward TNF-α with different concentrations in pH 7.4 PBS containing 0.1 mM Fc and 2 mM H_2O_2 at an applied potential of 0.05 V vs Ag/AgCl. Error bars represent the standard deviation, n = 3 (i.e., three individually prepared electrodes).

aryldiazonium salt modified surfaces, which can be attributed to direct attachment of aryldiazonium cations to the surface or to the as-grafted layers without losing nitrogen.[42] The surface ratio of PPC:PBA was calculated to be 8.1:1 by using atomic concentration of P 2p and −COOH (eq 1), which is close to their molar ratio of 9:1 in deposition solution. This can be attributed to their similar electrochemical reactivity during the electrografting process. The reduction potential plays a key role in determining the surface ratio of mixtures of aryldiazonium salts. The aryl diazonium salt with the more positive reduction potential typically dominates on the surface regardless of the ratio in solution.[43] For example, in our recent study, a surface ratio of 6:1 was obtained even though the molar ratio of the aryldiazonium salts PPC (reduction potential −0.55 V vs Ag/AgCl) and carboxyphenyl (reduction potential −0.25 V vs Ag/AgCl) in solution was 10 000:1.[23]

In Situ Antifouling Evaluation of Sensing Interface. The antifouling behavior of the final sensing interface (HRP-Ab2/TNF-α/Ab1/PPC−PBA/ITO) was investigated using cyclic voltammetry and impedance spectroscopy based on blocking effect and charge transfer resistance (R_{ct}), respectively. To make the sensor evaluation more compatible with analysis of actual human fluids, solutions containing HSA were employed as biofouling. In the in situ anti-biofouling test, the R_{ct} of final sensing interface (77.9 ± 9.8 Ω cm^2) was measured first in redox probe solution of $[Fe(CN)_6]^{3-/4-}$, followed by examining R_{ct} instantly after replacement with the redox probe solution containing HSA (1 mg/mL). As shown in Figure 3b, the R_{ct} value increased slightly to 82.4 ± 12.5 Ω cm^2; however, the peak separation and peak current were held almost constant. Even after the electrodes were held stationary in the HSA solution for 1 h, the R_{ct} value was found to increase

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slightly to 104.6 ± 9.8 Ω cm^2, while the peak separation and peak current in the cyclic voltammogram (Figure 3a) remained almost unchanged. The above results indicated that the sensing interface represents a high tolerance toward high-concentration nonspecific protein adsorption (only trace amount of protein can be adsorbed) in a long time scale.

Analytical Performance of the Immunosensor for the Electrochemical Detection of TNF-α. The performance of the immunosensor for TNF-α was investigated relative to the analytical criteria described in the introduction. As shown in Figure 4a, the amperometric response of the immunosensor increased with increasing concentration of TNF-α. At each concentration of TNF-α, each measurement was performed in triplicate using an independently prepared electrode for every single. The calibration curve (Figure 4b) was obtained from the chronoamperograms from the stable current response at 300 s, and presented a good linear relationship between the current response and the logarithm of TNF-α concentrations within the range of 0.01−500 ng/mL. The range was much broader than the data (1−1000 pg/mL) reported by Kongsuphol and co-workers, in which magnetic beads conjugated with different ligands were used for removal of nonspecific protein and electrochemical detection.[21] The linear regression equation I = 11.18 lg [TNF-α] + 120.9 was established with a correlation coefficient of 0.9903. The lowest detected concentration was 10 pg/mL. This is similar to a previously reported value using a alkaline phosphatase functionalized gold nanoparticles/poly-(styrene-*co*-acrylic acid) composite based electrochemical immunoassay.[36]

With our stated goal of developing an electrochemical immunosensor that can operate in complex biological fluids, the selectivity of the chronoamperometric response was studied with regard to proteins such as hemoglobin (Hb), human serum albumin (HSA) that might nonspecifically adsorb onto the sensing interface. The study was implemented by assaying the current response toward the low-concentration (0.5 ng/mL) target TNF-α in the presence of physiologically relevant concentrations (5 ng/mL) of potential proteins that could nonspecifically adsorb onto the sensing interface. As shown in Figure 5 (first three bars on the left), the current signal from interference proteins (HSA or Hb) is similar to that of negative control (PBS as blank sample) but much lower than the signal from target TNF-α. More importantly, the current signal (four bars on the right) from mixture of TNF-α and interfering proteins showed comparable results to that of TNF-α alone, indicating that the immunosensor exhibits high specificity toward TNF-α with limited influence from other nonspecifically adsorbed proteins.

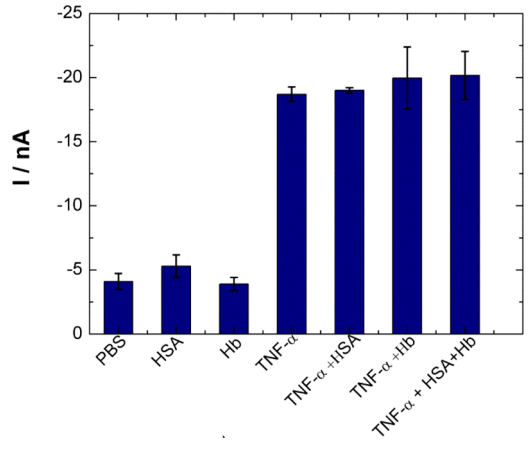

Figure 5. Selectivity of the proposed mixed-layer based immunosensor to 0.5 ng/mL TNF-α by comparing it to the interfering proteins at 5 ng/mL level: HSA and hemoglobin (Hb). Error bars represent the standard deviation, n = 3 (i.e., three individually prepared electrodes).

Analysis in Blood. With the excellent performance of the electrochemical immunosensor in the presence of interfering proteins, the next step was to challenge the device in blood samples. The results obtained from the electrochemical immunosensor were correlated with the analyses performed using a commercially available ELISA kit as shown (Table 1).

Table 1. Comparison of TNF-α Levels in Whole Blood Detected by Electrochemical Assay and Commercial ELISA Method

blood samples	1	2	3
Immunosensor (pg/mL)[a]	22.0	18.8	14.9
ELISA kit (pg/mL)[a]	19.7	17.9	15.3
Relative deviation (%)[b]	11.7	5.0	2.6

[a]The average value of three determinations (i.e., three individually prepared electrodes or ELISA tests). [b]Relative deviation = ([TNF-α]$_{immunosensor}$ − [TNF-α]$_{ELISA\ kit}$)/ [TNF-α]$_{ELISA\ kit}$

The relative deviation between the electrochemical immunosensor and the ELISA was between 2.6% and 11.7%. These deviations are quite small and reveal that the experimental data from the developed immunosensor correlated well with those from the commercial ELISA kit. Therefore, the proposed immunosensor could be promising for the clinical determination of TNF-α.

CONCLUSION

This paper describes an electrochemical immunosensor with integration of PPC antifouling layer and PBA layer for anchoring "sandwich" component of capture antibody-TNF-α-HRP conjugated detection antibody. This mixed-layer based immunosensor allows sensitive and specific detection of vital antigen of TNF-α with a wide dynamic range (0.01−500 ng/mL) and minimum detection level of 10 pg/mL. The antifouling behavior benefiting from zwitterionic PPC is highlighted as its tolerance of extremely high concentration of nonspecific protein (1 mg/mL of HSA). The developed immunosensor was applied to analyze nonpretreated whole blood samples; the results were comparable to that obtained from a commercial ELISA kit, implying its great potential for future diagnosis of biological samples in the clinic. The mixed-layer based sensing platform is versatile and can be extended to detect other biomarkers of interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.6b00532.

Additional figure represents the electrochemical response to hydrogen peroxide at the immunosensor: S-1(a) depicts current response of the developed immunosensor in the absence and presence of H_2O_2; S-1(b) illustrates the dependence of the chronoamperometric reduction current on the concentration of H_2O_2 for the PPC−PBA mixed layer-based immunosensor. (PDF)

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Notes

The authors declare no competing financial interest.

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